

**COMPOSITIONS AND METHODS FOR THE TREATMENT
AND CLINICAL REMISSION OF PSORIASIS**

This application is a continuation of U.S. patent application serial number 09/809,003,
5 filed March 16, 2001, now U.S. Patent No.

Field of the Invention

The present invention relates generally to immunotherapeutic agents or therapeutic
agents, compositions comprising those agents, and methods of use of those agents and
10 compositions for the treatment and clinical remission of psoriasis.

Background

Psoriasis is a chronic, genetically-influenced, remitting and relapsing scaly and
inflammatory skin disorder of unknown etiology that affects 1 to 3 percent of the world's
population. There are several types of psoriasis, including plaque, pustular, guttate and
15 arthritic variants. There is at present no cure for psoriasis, but rather only suppressive
therapy (Greaves and Weinstein, 1995, Drug Therapy, 332: 581-588). Indications for
treatment may arise from local symptoms, for example, pain, itching, reduction of manual
dexterity, severe problems with walking, and cosmetic problems such as prominent hand, leg,
or facial lesions. Due to the toxicity of the available therapies, patients with limited disease
20 will often decide to forego treatment beyond the avoidance of provoking factors.

The goal of current treatments has been to decrease the severity and extent of
psoriasis to the point at which it no longer interferes substantially with the patient's
occupation, well-being, or personal or social life. The initial treatment for stable plaque
psoriasis of any severity is topical. In patients in which more than 20 percent of the skin is
25 affected, however, topical treatment alone may be impractical and systemic therapy may also
be indicated at the outset.

The topical treatment for plaque psoriasis incorporates the use of emollients, keratolytic agents, coal tar, anthralin, corticosteroids of medium to strong potency, and calpotriene. All of these treatments have variable efficacy, fail to prevent frequent relapses of the disease, exhibit side effects, and pose cosmetic problems of their own.

Summary of the Invention

The present invention provides compositions and methods for the treatment and clinical remission of psoriasis. In one aspect, polypeptides derived from particulate antigens of protozoans of the genus *Leishmania* are provided that can generate an immune response in an individual resulting in abatement of the clinical symptoms of psoriasis. In one embodiment of this aspect, the polypeptides comprise at least one of the amino acid sequences recited in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, and SEQ ID NO:14, or immunogenic variants thereof. An immunogenic variant of an amino acid sequence may be either a truncated version of the sequence that retains a substantial amount of the activity of the original, or an

altered version of the sequence retaining such activity and having conservative amino acid substitutions and/or modifications.

In another embodiment of this aspect, the polypeptides comprise at least an immunogenic portion of an amino acid sequence of a protein, or an immunogenic variant thereof, the protein isolated from protozoans of the genus *Leishmania* and having an apparent molecular weight of 21, 33, 44, 50, 55, 58, 65, or 77 kDa as determined by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

In yet another embodiment of this aspect, the polypeptides comprise at least an immunogenic portion of an amino acid sequence of a protein, or an immunogenic variant thereof, the protein isolated from protozoans of the genus *Leishmania* and having an apparent molecular weight after total reduction and alkylation of 73, 80, or 82 kDa as determined by SDS-PAGE.

In related aspects, the present invention provides nucleic acid sequences encoding such polypeptides, vectors incorporating such nucleic acid sequences, methods for the production of such polypeptides by transformation, transfection, or transduction of microbial host cells, and microbial host cells transformed by such vectors, transfected by such nucleic acid sequences, or transduced with such nucleic acid sequences.

In another aspect of the invention, immunotherapeutic agents incorporating one or more polypeptides of the present invention are provided. These immunotherapeutic agents may be polyvalent or monovalent, and may incorporate an adjuvant such as alumina to enhance the immune response obtained from an inoculated subject. In a preferred embodiment, the polyvalent immunotherapeutic agent contains polypeptide isolates from a mixture of four species of *Leishmania*, namely, *L.(L)amazonensis*, *L.(L)venezuelensis*, *L.(V)brasiliensis*, and *L.(L)chagasi*. Also in a preferred embodiment, the monovalent immunotherapeutic agent contains a polypeptide isolate from one of these four species.

In another aspect of the invention, methods for the treatment and clinical remission of

psoriasis are provided. In one embodiment, such a method involves administration of a therapeutically effective amount of a pharmaceutical composition comprising one or more polypeptides of the present invention to a subject in order to induce an immune response resulting in abatement of the clinical symptoms of psoriasis. In a related embodiment, the method involves administration of a therapeutically effective amount of a pharmaceutical composition comprising one or more nucleic acid sequences of the present invention to a subject. The term "therapeutically effective" as used herein means a reduction of approximately 70-100% of Psoriasis Area and Severity Index as is understood by those skilled in the art.

Other aspects of the invention include the use of the nucleic acid sequences of the invention as probes for genetic analysis and as nucleic acid molecular weight markers, and the use of the polypeptides of the invention as molecular weight markers.

Detailed Description of the Invention

The present invention concerns novel compositions and methods for the treatment and clinical remission of psoriasis. The compositions comprise immunogenic polypeptides or the nucleic acids encoding them. The polypeptides of the present invention can elicit an immune response in a warm-blooded animal, thereby inducing clinical remission of psoriasis. As used herein, the term "warm-blooded animal" includes humans. In one embodiment of the invention, the subject polypeptides can be isolated from *Leishmania* protozoa and, preferably, from killed *Leishmania* amastigote protozoa. The polypeptides of the subject invention can be obtained from protozoa of the *Leishmania* genus using standard protein isolation procedures which are known in the art. Also contemplated by the present invention are immunotherapeutic agents and pharmaceutical compositions incorporating the immunogenic polypeptides of the present invention. In one embodiment, a first-generation polyvalent immunotherapeutic agent is provided, comprising a polypeptide isolate of a mixture of a plurality of *Leishmania* species, such as *L.(L)amazonensis*, *L.(L)venezuelensis*,

L.(V)brasiliensis, *L.(L)chagasi*, *L.(L)donovani*, *L.(L)infantum*, *L.(L)major*, *L.(L)panamensis*,
L.(L)tropica, and *L.(L)guyanensis*. Preferably, the mixture comprises *L.(L)amazonensis*,
L.(L)venezuelensis, *L.(V)brasiliensis*, and *L.(L)chagasi*. Most preferably, the mixture
consists of these four species. The organisms are preferably cultivated in the amastigote
stage in the synthetic culture medium specified in Table 1, supplemented with 5% fetal
bovine serum, typically at about 30-34° C. Subsequently, and during the stationary phase of
growth, the amastigotes are subjected to a medium containing an amount of N-p-tosyl-L-
Lysine chloromethyl ketone (TLCK) or a pharmacologically acceptable salt thereof effective
to kill the cells. The dead cells are then isolated and treated with the non-ionic detergent
Nonidet p-40 (NP40) to solubilize the surface antigens, which are discarded. The particulate
antigens that comprise the immunogenic polypeptides of the present invention can be
collected by centrifugation following cell disruption. These polypeptides are washed with
phosphate-buffered saline (PBS) and subsequently resuspended by sonication for 5 minutes at
4° C in PBS containing alumina.

In another embodiment, a first-generation monovalent immunotherapeutic agent is
described, comprising a polypeptide isolate of a single *Leishmania* species chosen from the
group consisting of *L.(L)amazonensis*, *L.(L)venezuelensis*, *L.(V)brasiliensis*, *L.(L)chagasi*,
L.(L)donovani, *L.(L)infantum*, *L.(L)major*, *L.(L)panamensis*, *L.(L)tropica*, and *L.(L)*
guyanensis. Preferably, the single *Leishmania* species is chosen from the group consisting of
L.(L)amazonensis, *L.(L)venezuelensis*, *L.(V)brasiliensis*, and *L.(L)chagasi*. Procedures for
the preparation of this immunotherapeutic agent are otherwise identical to those disclosed
above for the first-generation polyvalent immunotherapeutic agent.

In another embodiment, a second-generation polyvalent immunotherapeutic agent is
described, comprising a polypeptide isolate of a mixture of a plurality of *Leishmania* species,
such as *L.(L)amazonensis*, *L.(L)venezuelensis*, *L.(V)brasiliensis*, *L.(L)chagasi*, *L.(L)donovani*,
L.(L)infantum, *L.(L)major*, *L.(L)panamensis*, *L.(L)tropica*, and *L.(L) guyanensis*. Preferably,

the mixture comprises *L.(L)amazonensis*, *L.(L)venezuelensis*, *L.(V)brasiliensis*, and *L.(L)chagasi*. Most preferably, the mixture consists of these four species. The organisms are preferably cultivated in the amastigote stage in the synthetic culture medium specified in Table 1, supplemented with 5% fetal bovine serum, typically at about 30-34° C.

- 5 Subsequently, and during the stationary phase of growth, the amastigotes are subjected to a medium containing an amount of N-p-tosyl-L-Lysine chloromethyl ketone (TLCK) or a pharmacologically acceptable salt thereof effective to kill the cells. The dead cells are then isolated and treated with the non-ionic detergent Nonidet p-40 (NP40) to solubilize the surface antigens, which are discarded. The particulate antigens that comprise the
- 10 immunogenic polypeptides of the present invention can be collected by centrifugation following cell disruption. These polypeptides are washed with phosphate-buffered saline (PBS) and subsequently resuspended by sonication for 5 minutes at 4° C in 8 M Urea, 0.025 M Tris (Tris-hydroxy-methyl-amino-methane). The polypeptides are then subjected to chromatography on a DEAE-Sephadex column with a stepwise elution from 0.05-0.3 M NaCl
- 15 in a solution containing 8 M Urea, .025 M Tris, pH 8.3. Seven protein fractions are collected, and an inoculum comprising each protein fraction is made by resuspending the polypeptides of each fraction in PBS containing alumina.

- In another embodiment, a second-generation monovalent immunotherapeutic agent is described, comprising a polypeptide isolate of a single *Leishmania* species chosen from the
- 20 group consisting of *L.(L)amazonensis*, *L.(L)venezuelensis*, *L.(V)brasiliensis*, *L.(L)chagasi*, *L.(L)donovani*, *L.(L)infantum*, *L.(L)major*, *L.(L)panamensis*, *L.(L)tropica*, and *L.(L)guyanensis*. Preferably, the single *Leishmania* species is chosen from the group consisting of *L.(L)amazonensis*, *L.(L)venezuelensis*, *L.(V)brasiliensis*, and *L.(L)chagasi*. Procedures for the preparation of this immunotherapeutic agent are otherwise identical to those disclosed
- 25 above for the second-generation polyvalent immunotherapeutic agent.

Alternatively, the subject polypeptides can be synthesized according to known procedures and techniques, or produced recombinantly by transforming a host cell with one or more of the nucleotide sequences encoding the desired polypeptides. The polypeptides can be expressed in the host cell such that they can be isolated and purified to a desired degree of purification. The subject polypeptides can be used in accordance with the subject invention as a third-generation immunotherapeutic agent to treat psoriasis.

The instant invention further concerns nucleic acid sequences that can be useful in transforming appropriate host cells to cause them to produce the polypeptides of the invention; in administration to a warm-blooded animal, either directly or as part of a pharmaceutically-acceptable composition, to generate an immune response and thereby induce clinical remission of psoriasis in the animal; as labelled probes for genetic analysis; or as nucleic acid molecular weight markers.

One of ordinary skill in the art of molecular biology can obtain nucleic acids encoding the polypeptides of the present invention in view of the teachings provided herein. For example, the polypeptides of the first-generation immunotherapeutic agent of the present invention have been isolated and purified from protozoa of the *Leishmania* genus and comprise eight bands, identified by SDS-PAGE, representing eight distinct polypeptides having apparent molecular weights of 21, 33, 44, 50, 55, 58, 65, and 77 kDa, respectively. Each of these bands represents a separate polypeptide that can be isolated and sequenced in accordance with standard amino acid sequencing procedures. The polypeptides of each second-generation immunotherapeutic agent were purified by subjecting the first-generation immunotherapeutic agent containing the mixture of eight polypeptides to chromatography on diethylaminoethyl(DEAE)-Sephadex. Two fractions having all the activity to cure psoriasis were isolated and totally reduced and alkylated by standard procedures. These fractions were subjected to electrophoresis on acrylamide gels to separate the constituent polypeptides, and the amino acid sequence of each polypeptide was obtained by standard protein sequencing

procedures. The nucleotide sequences encoding each of these polypeptides can be derived from these amino acid sequences by application of the genetic code.

Additionally, the present invention contemplates the production of large quantities of the immunogenic polypeptides of the invention via introduction of the nucleic acids encoding them to microbial host cells. The nucleic acids can be introduced directly into the genome of the host cell or can first be incorporated into a vector which is then introduced into the host. Exemplary methods of direct incorporation include transduction by recombinant phage or cosmids, transfection where specially treated host bacterial cells can be caused to take up naked phage chromosomes, and transformation by calcium precipitation. These methods are well known in the art.

Exemplary vectors include plasmids, cosmids, and phages. A genomic library for a *Leishmania* species can be created by routine means, and DNA of interest isolated therefrom. For example, DNA of *Leishmania* protozoa can be isolated and restricted with known restriction enzymes. The resulting DNA fragments can then be inserted into suitable cloning vectors for introduction to a compatible host. Depending on the contemplated host, the vector may include various regulatory and other regions, usually including an origin of replication, one or more promoter regions, and markers for the selection of transformants. In general, the vectors will provide regulatory signals for expression and amplification of the DNA of interest.

Various markers may be employed for the selection of transformants, including biocide resistance, particularly to antibiotics such as ampicillin, tetracycline, trimethoprim, chloramphenicol, and penicillin; toxins, such as colicin; and heavy metals, such as mercuric salts. Alternatively, complementation providing an essential nutrient to an auxotrophic host may be employed.

Hosts which may be employed according to techniques well known in the art for the production of the polypeptides of the present invention include unicellular microorganisms,

such as prokaryotes, i.e., bacteria; and eukaryotes, such as fungi, including yeasts, algae, protozoa, molds, and the like, as well as plant cells, both in culture or in planta. Specific bacteria which are susceptible to transformation include members of the Enterobacteriaceae, such as strains of *Escherichia coli*; *Salmonella*; Bacillaceae, such as *Bacillus subtilis*;

5 *Pneumococcus*; *Streptococcus*; *Haemophilus influenzae*, and yeasts such as *Saccharomyces*, among others. As used herein, the term microbial host cell encompasses all of these prokaryotic and eukaryotic organisms, including plant cells, both in culture and in planta.

Universal probes can be obtained which hybridize with certain of the fragments of a DNA library, allowing identification and selection (or “probing out”) of the genes of interest,
10 i.e., those nucleotide sequences which encode the polypeptides described as part of the present invention. The isolation of these genes can be performed using techniques which are well known in the art of molecular biology. The isolated genes can be inserted into appropriate vectors for use in the transformation of microbial host cells. In addition, these genes can be subjected to standard nucleic acid sequencing procedures to provide specific
15 information about the nucleotide sequence of the genes encoding the subject polypeptides.

It is now well known in the art that when synthesizing a gene for improved expression in a host cell it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell. For purposes of the subject invention, “frequency of preferred codon usage” refers to the preference exhibited by
20 a specific host cell in usage of nucleotide codons to specify a given amino acid. To determine the frequency of usage of a particular codon in a gene, the number of occurrences of that codon in the gene is divided by the total number of occurrences of all codons specifying the same amino acid in the gene. Similarly, the frequency of preferred codon usage exhibited by a host cell can be calculated by averaging frequency of preferred codon
25 usage in a large number of genes expressed by the host cell. It is preferable that this analysis be limited to genes that are highly expressed by the host cell.

Thus, in one embodiment of the subject invention, bacteria, plants, or other cells can be genetically engineered, e.g., transformed with genes from protozoa of the *Leishmania* spp., to attain desired expression levels of the subject polypeptides or proteins. To provide genes having enhanced expression, the DNA sequence of the gene can be modified to comprise

5 codons preferred by highly expressed genes to attain an A+T content in nucleotide base composition which is substantially that found in the transformed host cell. It is also preferable to form an initiation sequence optimal for said host cell, and to eliminate sequences that cause destabilization, inappropriate polyadenylation, degradation and termination of RNA and to avoid sequences that constitute secondary structure hairpins and RNA splice sites. For

10 example, in synthetic genes, the codons used to specify a given amino acid can be selected with regard to the distribution frequency of codon usage employed in highly expressed genes in the host cell to specify that amino acid. As is appreciated by those skilled in the art, the distribution frequency of codon usage utilized in the synthetic gene is a determinant of the level of expression.

15 Assembly of the genes of this invention can be performed using standard technology known in the art. A structural gene designed for enhanced expression in a host cell can be enzymatically assembled within a DNA vector from chemically synthesized oligonucleotide duplex segments. The gene can then be introduced into the host cell and expressed by means known in the art. Preferably, the protein produced upon expression of the synthetic gene is

20 functionally equivalent to a native protein. According to the subject invention, "functionally equivalent" refers to identity or near identity of function. A synthetic gene product which has at least one property relating to its activity or function that is similar or identical to a natural protein is considered functionally equivalent thereto.

It is also well known in the art that the nucleotide sequences of the subject invention

25 can be truncated such that certain of the resulting fragments of the original full-length sequence can retain the desired characteristics of the full-length sequence. A wide variety of

restriction enzymes are well known by those skilled in the art to be suitable for generating fragments from larger nucleic acid molecules. For example, it is well known that Bal31 exonuclease can be conveniently used for time-controlled limited digestion of DNA. See, for example, Maniatis *et al.* (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, pages 135-139. See also Wei *et al.* (1983) *J Biol. Chem.* 258:13006-13512. Thus, Bal31 exonuclease (commonly referred to as “erase-a-base” procedures) allows for the removal of nucleotides from either or both ends of the subject nucleic acids, consequently generating a wide spectrum of fragments, many of which encode products that are functionally equivalent to the natural polypeptide sequences of the present invention. Labeling procedures are also well known, and the ordinarily skilled artisan could routinely screen the labeled fragments for their hybridization characteristics to determine their utility as probes. For example, it is routine to label nucleic acids for use as specific and selective probes in genetic identification or diagnostic procedures. A person of ordinary skill in the art would recognize that variations or fragments of those sequences, which specifically and selectively hybridize to the DNA of *Leishmania* spp., could also function as a probe. It is within the ordinary skill of persons in the art, and does not require undue experimentation, to determine whether a segment of the subject nucleic acids is a fragment or variant which specifically and selectively hybridizes in accordance with the subject invention. Therefore, fragments or variants of these nucleic acids can be useful as probes to identify, diagnose, or distinguish *Leishmania* species.

It would also be recognized that the polynucleotides or peptides of the subject invention can be useful as molecular weight markers in respective nucleic acid or amino acid molecular weight determinations or assays.

In order to obtain a first-generation immunotherapeutic agent according to the subject invention, organisms of the genus *Leishmania* can be cultivated in synthetic culture medium comprising the ingredients listed in Table 1. In a preferred embodiment, the culture medium

is supplemented with 5% fetal bovine serum. Cultivation of the protozoa according to the subject invention is typically carried out at about 30-34°C. In a particularly preferred embodiment, cultivation of the protozoa is carried out in the amastigote stage of its life cycle.

5 **Table 1: Leishmania culture medium.**

Ingredient	mg/lit	Ingredient	mg/lit
Methionine	140	Carnosine	25
Tryptophan	50	Citrulline	50
α -Amino Adipic Acid	3	Sarcosine	57
Asparagine	165	CaCl ₂	265
Cystine	47	Fe(NO ₃) ₉ H ₂ O	0.72
Histidine	6	KCl	400
Aspartic Acid	120	MgSO ₄ 7 H ₂ O	200
Alanine	512	NaCl	5,850
Proline	248	NaHCO ₃	2,000
Lysine	337	NaH ₂ PO ₄ H ₂ O	140
Taurine	6	Tricine	900
Isoleucine	191	Hemin	1
Ornithine	3	HEPES	2,000
Tyrosine	210	Glucose	1,000
β -alanine	80	D-ribose	10
Phosphoserine	23	2-Deoxy-ribose	10
α -amino Butyric Acid	8	Cholecalciferol(D ₃)	0.1
Leucine	440	Biotin	1
Arginine	413	Pyridoxamine	0.05
Serine	220	Pyridoxal	1
Hydroxylysine	12	Cyanocobalamin(B ₁₂)	0.01
Glutamine	164	Choline	1
Glutamic Acid	420	Thiamine (B ₁)	1
Cysteine	0.5	Inositol	2
Phosphoethanolamine	25	α -Tocopherol	0.01
Threonine	200	3-phytylmenadione(K ₁)	0.01
Glycine	235	Menadione (K ₃)	0.01
Phenylalanine	240	Retinol (A)	0.14
Valine	266	Riboflavin (B ₂)	0.1
d-Pantothenic Acid	1	6,8 Thiotic Acid	0.01
Ascorbic Acid	0.05	Pyridoxine (B ₆)	0.025
p-Aminobenzoic Acid	0.05	Folic Acid	1
Ergocalciferol (D ₂)	0.1	Niacinamide	1
L-carnitine	0.05	Tetrahydrofolic Acid	0.5
DL-methionine-S-methyl-sulfonium chloride (U)	0.05	Adenosine-5-Triphosphate (ATP)	5.5
2-Deoxyadenylic acid (d-AMP)	3.0	2'-Deoxyuridine-5-monophosphate (d-UMP)	3.0
5'-Thymidylic Acid (TMP)	3.0	5'-Deoxyguanylic Acid (d-GMP)	3.0
2'Deoxycytidine-5-	3.0	Hydroxyproline	262.5

The culture medium comprising the protozoan cells can then be treated in order to inactivate, and preferably kill, the cells. Upon isolation of those cells, the antigenic proteins can be purified therefrom and included in a pharmaceutically acceptable carrier, e.g., buffer solution, to create a second-generation immunotherapeutic agent. Preferably, the cells are inactivated or killed with a non-lysing agent, e.g., TLCK. The antigenic proteins of the present invention are particulate proteins that can be isolated from the cells using accepted methods. In a more specific embodiment the method of creating the second-generation immunotherapeutic agent of the present invention comprises the steps of (1) cultivating protozoa, preferably in the amastigote stage, in an appropriate culture medium; (2) treating said protozoan cells to inactivate or kill the cells; (3) isolating the treated cells; (4) extracting antigenic proteins from the isolated cells; and (5) formulating the second-generation immunotherapeutic agent composition by combining one or more isolated antigenic proteins with a pharmaceutically acceptable carrier, e.g., phosphate buffered saline (PBS). A preferred pharmaceutically acceptable carrier is a PBS solution having alumina present within the solution.

To cure psoriasis in patients with clinical and histopathological diagnosis of the disease, the first-generation polyvalent immunotherapeutic agent was administered intramuscularly, in the deltoid region, once a month, once every 15 days or once a week according to disease severity, for 7.6 ± 6.0 months on average, at 500 $\mu\text{g}/\text{dose}$.

Furthermore to cure psoriasis a monovalent immunotherapeutic agent with each one of the *Leishmania spp.* present in the first-generation polyvalent immunotherapeutic agent was used as a subject composition with similar results to the polyvalent immunotherapeutic agent.

Furthermore to cure psoriasis a second-generation immunotherapeutic agent containing the protein fractions isolated by chromatographic means from the crude first-generation immunotherapeutic agent together with 0.1 ml alumina/mg protein was administered intramuscularly in the deltoid region once every 15 days for 3-4 doses at 200 µg/dose in 0.5 ml.

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1

Preparation of the Immunogen

Organisms of the genus *Leishmania* are cultivated in the amastigote stage in the synthetic culture medium specified in Table 1, supplemented with 5% fetal bovine serum typically at about 30-34°C (O'Daly *et al.*, 1988, *Acta Tropica (Basel)*, Vol. 45, pp. 109-126). For the second-generation immunotherapeutic agent, amastigotes at the stationary phase of growth were collected by centrifugation (800 xg for 20 minutes at 4°C), washed in Phosphate Buffered Saline (PBS), and incubated for 3 days at 30-34° C in Eagle's MEM (Gibco) containing 150 µg of TLCK to inactivate the parasites as described (O'Daly *et al.*, 1986, *Acta Tropica (Basel)*, Vol. 43, pp. 225-236). After two washes with PBS (12.100 xg for 10 minutes at 4°C) 1×10^8 parasites/ml were incubated in MEM containing 0.12% Nonidet-P-40 (NP40, Sigma) for 30 minutes at 4°C to solubilize the surface antigens which were discarded (O'Daly *et al.*, 1990 *AM J Trop. Med. Hyg.*, Vol. 43, pp. 44-51). Particulate antigens were collected by centrifugation (12.100 xg for 10 minutes at 4°C), washed twice with PBS and sonicated for 5 minutes at 4°C in a Sonifier Cell Disrupter (Model WI 85, Heath-Systems-Ultrasonic, Inc., Plainview, New York) at the microtip limit of the output control at 50W. Protein content was determined by the method of Lowry (Lowry, 0. *et al.*, 1951, *J Biol.*

Chem., Vol. 193, pp. 265-275). The final monovalent first generation immunogen preparation contained 1 mg/ml of each *Leishmania* spp. antigens in PBS containing alumina (Aluminum hydroxide low viscosity gel REHYDRAGEL, Reheis Inc., New Jersey) at a concentration of 0.1 ml/mg (v/w) of parasite protein. Each step in the preparation of the immunogen was checked for sterility.

In another embodiment of the subject invention, particulate antigens were collected by centrifugation (12,100 xg for 10 minutes at 4°C), washed twice with PBS, dissolved in a solution containing 8 Molar Urea, 0.025 Tris (Tris-hydroxy-methyl-amino-methane) and sonicated for 5 minutes at 4°C in a Sonifier Cell Disrupter (Model WI 85, Heath-Systems-Ultrasonic, Inc., Plainview, New York) at the microtip limit of the output control at 50W. Protein fractions were separated by DEAE-chromatography.

The second-generation immunotherapeutic agent was prepared with each one of the seven protein fractions isolated after DEAE-chromatography of the subject composition containing only one leishmania specie as for example *L.(V)brasiliensis* or any other leishmania specie present in the crude first-generation immunotherapeutic agent. Protein content was determined by the method of Lowry (Lowry, O. et al, 1951, *J Biol. Chem.*, Vol. 193, pp. 265-275). Each protein fraction was dissolved in PBS and sonicated for 5 minutes at 4°C in a Sonifier Cell Disrupter (Model WI 85, Heath-Systems-Ultrasonic, Inc., Plainview, New York) at the microtip limit of the output control at 50W. Subsequently each fraction was filter-sterilized through 0.20 µm Millipore® filters. The final immunogen preparation contained 400 µg/ml of each of the antigenic fractions in PBS containing alumina (Aluminum hydroxide low viscosity gel REHYDRAGEL, Reheis Inc., New Jersey) at a concentration of 0.1 ml/mg (v/w) of the protein fraction. Each step in the preparation of the second generation immunogen was also checked for sterility.

Aliquots were incubated in ESM containing 5% Fetal Bovine Serum (FBS, Gibco) and in agar plates containing 12.5% (w/v) Bacto-Peptone (Difco), 12.5% (w/v) yeast extract (Becton Dickinson), 3.75% (w/v), glucose, and 3.75% (w/v) BBL agar (Becton Dickinson). Samples were incubated for 72 hours at 37°C to detect fast growing bacteria and for 3 weeks at 26°C for slow growing bacteria and fungus. Each batch of the immunogen was controlled by SDS-polyacrylamide gel electrophoresis to ensure consistency in the pattern of *Leishmania* protein bands. Each batch from the first and second generation immunotherapeutic agents was also tested with E-TOXATE (Sigma) for the presence of pyrogens. The first-generation immunogen was stable at 4°C for at least 4 weeks.

Example 2

Protein Components of the Immunogen

From the immunogen preparations obtained from the procedures described in Example 1 above, eight protein bands were identified via SDS-polyacrylamide gel electrophoresis of the TLCK-treated NP-40-extracted amastigotes from *Leishmania(L)amazonensis*, *Leishmania(L)venezuelensis*, *Leishmania(V)brasiliensis*, and *Leishmania(L)chagasi*, with apparent molecular weights of 21, 33, 44, 50, 55, 58, 65, and 77 kDa. In untreated entire amastigote extracts between 28 and 30 bands with molecular weights ranging from 29 to 96 kDa were observed in each *Leishmania* species, and major bands of 29, 34, 43, 58, and 65 kDa were observed.

The immunogen preparations of the second-generation immunotherapeutic agent, which contain protein fractions 3 and 4 obtained after DEAE-chromatography and total reduction and alkylation, had three bands with molecular weights of 73, 80, and 82 kDa.

Example 3

Safety and Immunogenicity

The immunogenic composition comprising the proteins of the second-generation immunotherapeutic agent, described in Examples 1 and 2, above, was injected into a human

volunteer at monthly intervals, beginning with 50 µg and increasing the dose by 50 µg each month, in order to determine the dose capable of inducing an IDR greater than 5 mm. This dose was found to be 200 µg. At both one month and six months after the last dose of immunotherapeutic agent, the following blood tests were performed on this volunteer:

5 complete blood count; differential white blood cell count; urea; creatinin; sugar alkaline phosphatase; bilirubin; transaminases; cholesterol; triglycerides; C reactive protein; serological tests such as VDRL, HIV, antinuclear antibodies, LE cells; and urine and fecal analysis. All the values were within normal limits, and no side effects were observed.

Example 4

10 Preparation of Immunotherapeutic agent Compositions

For the first-generation monovalent immunotherapeutic agent, cultivated amastigotes of each species of *Leishmania* were collected by centrifugation (800xg for 20 minutes at 4°C), washed in Phosphate Buffered Saline (PBS) and incubated for 3 days at 30-34° C in Eagles's MEM (Gibco) containing 150 µg of TLCK to inactivate the parasites as described, at

15 1×10^8 parasites/ml. This step is preferably carried out when the amastigotes are in the stationary growth phase, after two washes with PBS (12.100 x g for 10 minutes at 4°C).

In a particularly preferred embodiment, preparation of a protective monovalent first generation immunogenic composition according to the subject invention comprises the following steps:

- 20 A) cultivating organisms of the genus *Leishmania* in the amastigote state in a synthetic culture medium containing the ingredients listed in Table 1 supplemented with 5% fetal bovine serum typically at about 30-34°C;
- B) subjecting organisms of the genus *Leishmania* in the amastigote stage, and at the stationary phase of growth, to a medium containing an amount of N-p-

tosyl-L-Lysine chloromethyl ketone or a pharmacologically acceptable salt thereof effective to kill said cells;

- C) isolating said killed cells;
- D) extracting the surface proteins with the non-ionic detergent Nonidet p-40;
- 5 E) centrifugation of the preparation to isolate particulate antigens;
- F) washing twice with PBS; and
- G) forming an immunizing inoculum comprising said particulate antigens from said killed cells by resuspending them in phosphate buffered saline comprising alumina.

10 For the second generation immunotherapeutic agent composition, cultivated amastigotes were collected by centrifugation (800xg for 20 minutes at 4°C), washed in Phosphate Buffered Saline (PBS) and incubated for 3 days at 30-34°C in Eagles's MEM (Gibco) containing 150 µg of TLCK to inactivate the parasites as described, at 1×10^8 parasites/ml. This step is preferably carried out when the amastigotes are in the stationary
15 growth phase, after two washes with PBS (12.100 x g for 10 minutes at 4°C).

In a particularly preferred embodiment, preparation of a protective second generation immunogenic composition according to the subject invention comprises the following steps:

- A) cultivating organisms of the genus *Leishmania* in the amastigote state in a synthetic culture medium containing the ingredients listed in Table 1
20 supplemented with 5% fetal bovine serum typically at about 30-34°C;
- B) subjecting organisms of the genus *Leishmania* in the amastigote stage and at the stationary phase of growth, to a medium containing an amount of N-p-tosyl-L-Lysine chloromethyl ketone or a pharmacologically acceptable salt thereof effective to kill said cells;
- 25 C) isolating said killed cells;

- D) extracting the surface proteins with the non-ionic detergent Nonidet p-40;
- E) centrifugation of the preparation to isolate particulate antigens;
- F) washing twice with PBS,
- G) dissolving in a solution containing 8 Molar Urea, 0.025 Molar Tris (Tris-
5 hydroxy-methyl-amino-methane) and sonicating for 5 minutes at 4°C in a
Sonifier Cell Disrupter (Model WI 85, Heath-Systems-Ultrasonic, Inc.,
Plainview, New York) at the microtip limit of the output control at 50W.
- H) separating protein fractions in a DEAE-Sephadex column with a NaCl
stepwise elution from 0.05-0.3 Molar NaCl concentration in a solution
10 containing 8 Molar Urea, 0.025 Molar Tris pH 8.3; and
- I) forming an immunizing inoculum comprising said particulate antigens from
said killed cells by resuspending them in phosphate buffered saline
comprising alumina.

In a particularly preferred embodiment, preparation of an immunogenic composition
15 for clinical remission of psoriasis according to the second-generation subject invention
comprises the following steps:

- A) cultivating organisms of the genus *Leishmania* in the amastigote state in a
synthetic culture medium containing the ingredients listed in Table 1
supplemented with 5% fetal bovine serum typically at about 34°C;
- 20 B) subjecting organisms of the genus *Leishmania* in the amastigote stage and at
the stationary phase of growth, to a medium containing an amount of N-p-
tosyl-L-Lysine chloromethyl ketone or a pharmacologically acceptable salt
thereof effective to kill said cells;
- C) isolating said killed cells;
- 25 D) extracting the surface proteins with the non-ionic detergent Nonidet p-40;

- E) DEAE Sephadex chromatography of particulate antigens from only one *Leishmania* specie, as for example *L.(V)brasiliensis* or any other *Leishmania* specie present in the first-generation immunotherapeutic agent;
- F) isolating seven protein fractions in 8 Molar urea, 0.025 Molar Tris pH 8.3, separated using stepwise elution with 0.05-0.3 Molar NaCl;
- G) dialysis vs distilled water and lyophilization of protein fractions;
- H) dissolving the protein fractions in phosphate buffered saline;
- I) determining protein content of the fractions by the method of Lowry (Lowry, 0. et al, 1951, *J Biol. Chem.*, Vol. 193, pp. 265-275);
- J) sonicating each protein fraction in phosphate buffered saline for 5 minutes at 4°C in a Sonifier Cell Disrupter (Model WI 85, Heath-Systems-Ultrasonic, Inc., Plainview, New York) at the microtip limit of the output control at 50W;
- K) passing each fraction through 0.20 µm Millipore® filters; and
- L) forming a second-generation immunizing inoculum comprising one or more of said protein fractions by resuspending the one or more fractions in phosphate buffered saline containing alumina.

Example 5

Treatment of Psoriasis With a First-Generation Polyvalent Immunotherapeutic Agent
Containing L.(L)amazonensis, L.(L)venezuelensis, L.(L)brasiliensis, and L.(L)chagasi.

TABLE 2: Age groups in the study population.

Age groups	Patients	%
[0-5]	8	0.29
[6-12]	65	2.35
[13-18]	90	3.25
[19-25]	268	9.68
[26-40]	997	35.99
[41-65]	1196	43.18

>65	146	5.27
Total	2770	100

The majority of patients (79.17%) were between 26-65 years of age with average age of 42.56 ± 26.11 years and a range between 1 and 88 years of age.

TABLE 3: Characteristics of the study population.

	PATIENTS	AGE	TIME (YEARS) WITH PSORIASIS	PATIENTS HAVING RELATIVES WITH PSORIASIS
Males	1545 (55.8%)	42.1±14.3	11.2±9.6	500 (32.3%)
Females	1225 (44.2%)	38.6±15.3	12.0±10.0	472 (38.5%)
Age ≤ 25	431 (15.6%)	18.7±5.5	6.1±4.8	172 (39.9%)
Age ≥ 26	2339 (84.4%)	44.6±12.4	12.6±10.2	800 (34.2%)
Total	2770	40.6±14.9	11.6±9.8	972 (35.0%)

35% had parents with psoriasis and the evolution time of the disease was 11.6±9.8

5 years, similar in males and females, with a range between 2 and 46 years.

Table 4: Clinical types of Psoriasis in the study population.

	PLAQUE	GUTATA	PLAQUE + GUTATA	PALM PLANTAR	ERYTHRO- DERMIA	INVERSE	PLAQUE + ARTHRITIS	NAILS
Male	1229 (56.1%)	67 (48.9%)	78 (56.9%)	37 (39.4%)	36 (72.0%)	14 (58.3%)	53 (55.2%)	29 (72.5%)
Female	963 (43.9%)	70 (51.1%)	59 (43.1%)	57 (60.6%)	14 (28.0%)	10 (41.7%)	43 (44.8%)	11 (27.5%)
Age ≤25	320 (14.6%)	33 (24.1%)	24 (17.5%)	19 (20.2%)	10 (20%)	3 (12.5%)	8 (8.3%)	5 (12.5%)
Age ≥26	1872 (85.4%)	104 (75.9%)	113 (82.5%)	75 (79.8%)	40 (80%)	21 (87.5%)	88 (91.7%)	35 (87.5%)
Total	2192 (79.1%)	137 (10.1%)	137 (10.1%)	94 (0.3%)	50 (1.8%)	24 (0.8%)	96 (3.4%)	40 (0.3%)

92.6 % had the clinical form of plaque psoriasis distributed in its pure form (79.1%)

or associated with guttata (10.1%) or arthritis (3.4%); 10.1% had the Guttata pure form; 0.3%

10 had the palmar and plantar form, 1.8% had Erythrodermia and 3.4% had psoriatic arthritis.

TABLE 5: Study population and response to vaccination in psoriatic patients distributed by gender and age.

PASI ¹ BEFORE IMMUNOTHERAPEUTIC AGENT			REDUCTION OF PASI ¹ AFTER VACCINATION ²					QUIT
			100%	99-70%	69-40%	39-10%	<10%	QUIT
Males	1545	18.5±16.9	323 (49.8%)	600 (57.0%)	185 (56.7%)	105 (61.8%)	55 (59.8%)	272 (56.5%)
Females	1225	13.7±14.9	325 (50.2%)	453 (43.0%)	141 (43.3%)	65 (38.2%)	37 (40.2%)	209 (43.5%)
Age ≤25	431	13.0±14.7	131 (20.2%)	150 (14.2%)	50 (15.3%)	24 (14.1%)	12 (13.0%)	69 (14.3%)
Age ≥26	2339	17.0±16.4	517 (79.8%)	903 (85.8%)	276 (84.7%)	146 (85.9%)	80 (87.0%)	412 (85.7%)
Total	2770	16.4±16.2	648 (28.0%)	1053 (46%)	326 (14,0%)	170 (7%)	92 (4%)	481 (17.4%)

5 ¹PASI=Psoriasis area and severity index
²Eight years of follow-up

Ninety six % of patients responded to treatment with a decrease in PASI values greater than 10%, and only 4% responded with a decrease in PASI values less than 10% from the initial PASI value before treatment. Twenty eight % had 100% remission of lesions, their disease disappeared completely, similar in males and females. Overall 74% had between 70-100% remission of lesions and 21% from 10-69% remission as compared with initial PASI values. 17.4% of volunteers quit treatment after 1-2 doses of immunotherapeutic agent (see below)

15 **Table 6: Comparison of immunotherapeutic agent doses in each clinical remission group.**

IMMUNOTHERAPEUTIC AGENT DOSES FOR REDUCTION OF PASI AFTER VACCINATION ¹							
		100%	99-70%	69-40%	39-10%	<10%	QUIT
Males	1545	7.7±6.5	11.3±10.8	9.2±10.2	5.9±4.5	6.1±4.8	1.6±1.1
Females	1225	7.5±5.6	10.6±10.0	8.8±8.7	6.0±4.6	5.9±5.0	1.5±1.1

Age ≤ 25	431	6.5±4.2	10.6±10.0	8.2±8.4	6.1±6.1	6.5±4.6	1.4±0.6
Age ≥ 26	2339	7.8±6.4	11.1±10.0	9.2±9.8	5.9±4.2	5.9±5.0	1.7±1.4
Total	2770	7.6±6.0	11.0±10.0	9.0±9.6	6.0±4.5	6.0±4.9	1.7±1.4

¹ Subjects' conditions were followed for eight years.

7.6±6.0 doses of immunotherapeutic agent were needed for 100% remission of psoriasis. The amount of doses in the groups with 70-90% and 40-69% remission were somewhat higher, reaching values of 11.0±10.0 and 9.0±9.6 respectively, which suggests that clinical remission depends mainly on the immunological response of the volunteer. The patient able to respond to the immunotherapeutic agent antigens is committed to do so since the beginning of treatment. The patient without response stays so, in spite of a higher number of immunotherapeutic agent doses.

10 **Table 7: Appearance of relapses after clinical remission of Psoriasis.**

APPEARANCE OF RELAPSES AFTER REMISSION IN 100% REMISSION GROUP									
Relapses	Initial PASI	Doses for 100% remission	Time ¹ for 100% remission	PASI at relapse	Time ¹ from remission to relapse	PASI at new remission	Doses for new remission	Time ¹ for new remission	% New remissions after relapse
188/648 (28.9%)	21.0±17.8	7.6±6.0	7.0±5.4	7.7±10.1	15.4±20.6	2.8±3.3	7.1±6.8	5.8±4.9	161/188 (85.6%)

¹ months

From the 648 patients with total remission of lesions 188 (28.9 %) volunteers had relapses of the disease after 15.4±20.6 months. PASI values at the time of relapse were 1/3 of the initial PASI value before treatment. The PASI at the new Clinical remission was considerable lower than the PASI at the time of relapse. The new remission occurred with 7.1±6.8 doses of immunotherapeutic agent after 5.8±4.9 weeks, a period of time lower than the time period observed in the first treatment cycle for Clinical remission of lesions. In this relapsing group 85.6% of patients had again remission of lesions after 6-7 doses of immunotherapeutic agent.

Table 8: Side effects after vaccination.

SIGNS AT THE SITE OF INOCULATION				SYSTEMIC SYMPTOMS	NONE
Pain	Heat	Redness	Nodule		
989(43.2%)	484(21.1%)	327(14.3%)	535 (23.4%)	588(25.7%)	1233(53.9%)

Minor side effects were observed at the site of inoculation in less than half of the patients with psoriasis, without difference due to gender or age. All of these disappeared within a few days. Results of the laboratory analysis of samples from 55 psoriasis patients who received 21.4 ± 13.1 doses of first-generation immunotherapeutic agent are shown in Table 9. All values were found to be within normal ranges.

Table 9: Laboratory analysis in 55 psoriasis patients with 21.4 ± 13.1 doses of first-generation immunotherapeutic agent.

White blood cell count/ul	6003 \pm 4165
% Neutrophiles	53.1 \pm 13.3
% Lymphocytes	29.3 \pm 13.3
% Monocytes	5.8 \pm 3.8
% Eosynophiles	2.9 \pm 2.3
% Basophiles	0.7 \pm 0.6
Red blood cell count x10 ⁶ /ul	4.7 \pm 0.6
Hemoglobin g/dl	13.3 \pm 1.9
Hematocrit(%)	42.0 \pm 5.9
VCM(fl)	91.6 \pm 7.7
MCH(pg)	29.2 \pm 3.2
MCHC(g/dl)	31.9 \pm 1.0
RDW-SD(fl)	20.1 \pm 14.9
Platelets x 10 ⁹ /ul	250.3 \pm 84.2
UREA(mg/dl)	19.7 \pm 8.5
CREATININE(mg/dl)	0.9 \pm 0.2
URIC ACID(mg/dl)	5.6 \pm 1.6
BLOOD SUGAR(mg/dl)	89.8 \pm 15.1
TOTAL PROTEIN(g/dl)	7.2 \pm 0.8
ALBUMINE(g/dl)	3.8 \pm 0.9
GLOBULINES(g/dl)	3.3 \pm 0.8
TRIGLICERIDES(mg/dl)	161.0 \pm 107.1
LOW DENSITY LIPOPROTEINS (mg/dl)	102.8 \pm 44.5
VERY LOW DENSITY LIPOPROTEINS (mg/dl)	35.0 \pm 23.3
LACTIC ACID DEHYDROGENASE (mg/dl)	36.1 \pm 13.2
PROTROMBIN TIME	11.7 \pm 1.3
TROMBOPLASTIN PARTIAL TIME	29.5 \pm 6.5

OXALOACETIC TRANSAMINASE(u/l)	29.0±14.1
PYRUVIC TRANSAMINASE(u/l)	26.1±15.1
SODIUM(mg/dl)	144.9±2.1
POTASSIUM(mg/dl)	4.2±0.3
CHLORINE(meq/l)	105.3±2.6
CALCIUM(mg/dl)	8.7±0.3
PHOSPHORUS(mg/dl)	2.9±0.4

Example 6

Trial of First-Generation Monovalent Immunotherapeutic agent

5 **Table 10: Follow-up of a single blind trial after injection of psoriasis patients with one of four Leishmania species present in the first-generation immunotherapeutic agent.**

	PASI BEFORE TREATMENT	IMMUNOT HERAPEU TIC AGENT DOSES	PASI AFTER TREATMENT	% PASI REDUCTION
LEISHMANIA SPECIE				
L.(L) amazonensis	6.4	3	1.4	78.1
L.(L) amazonensis	3.8	6	1.7	55.3
L.(L) amazonensis	3.6	3	1.4	61.1
L.(L) amazonensis	9.4	5	1.3	<u>86.2</u>
L.(L) amazonensis	2.3	3	0	<u>100.0</u>
L.(V) brasiliensis	36	2	15.4	57.2
L.(V) brasiliensis	11.9	2	1.8	<u>84.9</u>
L.(V) brasiliensis	13.9	5	6.4	54.0
L.(V) brasiliensis	5.8	4	1.9	67.2
L.(L) chagasi	2.8	5	0	<u>100.0</u>
L.(L) chagasi	52.2	3	0	<u>100.0</u>
L.(L) chagasi	10	3	4.5	55.0
L.(L) venezuelensis	15.6	3	5.3	66.0

Immunotherapeutic agents were also prepared using individual species of Leishmania from the first generation Immunotherapeutic agent and were subsequently tested for ability to induce Clinical remission of psoriasis lesions. The results in Table 15 clearly demonstrated

that it is not necessary to prepare a mixture of four *Leishmania* species in the first generation Immunotherapeutic agent to obtain clinical remission of lesions in psoriasis patients. One *Leishmania* species is as effective as the mixture of four species used in the polyvalent immunotherapeutic agent to induce lower PASI values up to 100% after treatment. Thus, in every leishmania extract, there is a factor that inhibits the inflammation associated with psoriasis.

Example 7

Formulation and Administration

The compounds of the invention are useful for various purposes, both therapeutic and non-therapeutic. Therapeutic application of the new compounds and compositions containing them can be contemplated to be accomplished by any suitable therapeutic method and technique presently or prospectively known to those skilled in the art. Further, the compounds of the invention have utility as starting materials or intermediates for the preparation of other useful compounds and compositions.

The dosage administered to a host in the above indications will be dependent upon the identity of the infection, the type of host involved, including the host's age, weight, and health, the existence and nature of concurrent treatments, if any, the frequency of treatment, and the therapeutic ratio.

The compounds of the subject invention can be formulated according to known methods for the preparation of pharmaceutical compositions. Formulations are described in detail in a number of sources which are well known and readily available to those skilled in the art. For example, *Remington's Pharmaceutical Science* by E.W. Martin describes formulations that can be used in connection with the subject invention. In general, the compositions of the subject invention will be formulated such that an effective amount of the bioactive compound(s) is (are) combined with a suitable carrier in order to facilitate effective administration of the composition.

Example 8

Chromatographic Separation of Protein Fractions from Leishmania Species and Blastogenic assay with Human Peripheral Blood Mononuclear Cells

Seven fractions were separated from the particulate *Leishmania chagasi* extract

5 (PP75), the first component of the first-generation immunotherapeutic agent, after treatment of the respective amastigote parasites with TLCK and extraction with NP-40 as mentioned previously.

The fractions were tested in a blastogenic assay with peripheral blood mononuclear cells from psoriatic patients before and after vaccination according to methods routinely used
10 in the art. For this example, 100 µl aliquots (triplicates) of each of the fractions dissolved in RPMI-1640 were pre-incubated in flat bottom microtiter plates (Falcon Plastics) with 2×10^5 peripheral blood mononuclear cells, separated in HISTOPAQUE (Sigma) and resuspended in 100 µl of RPMI-1640 containing 20% heat inactivated fetal bovine serum under methods routine in the art. Concanavalin A was used as positive control of lymphocyte stimulation.
15 48 hours latter, 0.2 µCi/well of ^3H -Thymidine was added in 10 µl aliquots and the samples were incubated for 18 additional hours. The cells were harvested on filter paper (Reeve Angel) using an automatic cell harvester (MASHII). The dried paper discs were placed in minivials with 2.5 ml Aquasol (NEN) and counted for 1 min. in a Packard Tri-Carb scintillation counter Model 3385. The stimulation index (S.I.) was calculated for each sample
20 by dividing the experimental counts per minute (c.p.m.) by the control c.p.m. (cultures with fractions or mitogens/control cultures in culture medium alone). The results are illustrated in Tables 11-14 below.

TABLE 11: Peripheral blood mononuclear cells blastogenesis with fractions from *L(L). chagasi* (PP75) before and after vaccination.

		BEFORE VACCINATION		CURED AFTER VACCINATION	
		n = 3		n= 5	
DEAE	ug	cpm/well	S.I.	cpm/well	S.I.

Sephadex	protein / well	X±SD	X±SD	X±SD	X±SD
Fraction 1	20	823±215	1.90±0.22	2044±1825	3.22±286
No NaCl	10	1297±835	2.81±1.5	1442±1425	2.59±276
	5	1587±1429	3.40±2.79	1424±1150	2.44±217
	2.5	627±282	1.40±0.41	1366±951	2.27±1.66
Fraction 2	20	908±103	2.22±0.79	2643±1798	4.36±2.96
0.05M NaCl	10	821±660	1.87±1.1	1880±1571	3.13±2.83
	5	761±324	1.73±0.49	1627±1137	2.75±2.05
	2.5	532±347	1.19±0.63	1129±900	1.94±1.7
Fraction 3	20	933±728	2.03±1.37	1735±1764	<u>3.03±3.4</u>
0.1M NaCl	10	941±552	2.08±1.77	1368±1528	<u>2.51±2.94</u>
	5	706±376	1.57±0.61	1360±1681	<u>2.45±3.23</u>
	2.5	717±632	1.57±1.21	1174±1382	<u>2.09±2.66</u>
Fraction 4	20	674±405	1.54±0.74	2514±1552	<u>4.25±2.73</u>
0.15M NaCl	10	600±305	1.38±0.55	1541±1548	<u>2.74±3.0</u>
	5	767±275	1.87±0.84	1330±1520	<u>2.36±2.93</u>
	2.5	940±346	2.35±1.29	1216±1225	<u>2.16±2.37</u>
Fraction 5	20	549±197	1.24±0.21	1411±1629	2.52±3.14
0.2M NaCl	10	472±181	1.48±0.58	1398±1562	2.49±3.01
	5	470±205	1.06±0.31	1095±1023	1.94±1.98
	2.5	353±112	0.87±0.03	1059±907	1.86±1.76
Fraction 6	20	726±126	1.70±0.12	1448±1127	2.52±2.17
0.25M NaCl	10	558±225	1.26±0.31	1354±818	2.46±1.77
	5	778±456	1.71±0.78	1280±752	2.28±1.52
	2.5	688±574	1.52±1.09	927±710	1.61±1.36
Fraction 7	20	694±325	1.54±0.48	1180±747	1.91±1.09
0.3 M NaCl	10	676±154	1.56±0.10	1608±1107	2.96±2.27
	5	604±217	1.39±0.31	1325±601	2.40±1.32
	2.5	580±315	1.28±0.52	1466±810	2.75±1.89
Concanavalin A	10	8452±7470	23.12±24.89	7988±2805	13.58±4.31
	5	22479±10642	55.05±29.29	28011±8183	52.67±22.89
Amastigote Parasites	4x10 ⁶	795±209	1.85±0.32	2099±1454	<u>3.40±2.02</u>
	2x10 ⁶	741±307	1.68±0.45	1725±1028	<u>2.75±0.99</u>
Culture medium		323±79	1.0±0.2	987±226	1.0±0.3

The group of patients before vaccination had S.I. > 1.0. These values increased markedly after vaccination. Results of the statistical analysis of both groups are as follows:

	Before vaccination	After vaccination
5 Parameter		
Mean	1.697143	2.571072
# points	28	28
Std deviation	.5298834	.6259645
Std error	.1001386	.1182962
10 Minimum	.87	1.61
Maximum	3.4	4.36
Paired t test:		
Mean difference =	-.8739286 (Mean of paired differences)	
95% confidence interval of the difference:	-1.150029 to -0.5978283	
15 Two-tailed p value is	< 0.0001 --- extremely significant-	

These results demonstrate that, after vaccination of psoriatic patients with any of the fractions of the *L.(L) chagasi* extract, lymphocytes are significantly stimulated. Higher stimulation index was observed with fractions 3 and 4 as well as live amastigotes.

20 Seven fractions were separated from the particulate *L(V) brasiliensis* extract (PMH27), a second component of the first-generation immunotherapeutic agent, after treatment of the respective amastigote parasites with TLCK and extraction with NP-40 as mentioned previously.

25 **TABLE 12: Peripheral blood mononuclear cells blastogenesis with fractions from *L.(V)brasiliensis* (PMH27) before and after vaccination.**

		BEFORE VACCINATION		BEFORE VACCINATION		AFTER VACCINATION	
		N=3 , S.I.< 1.0		N=2 , S.I.> 1.0		CURED, N = 3	
DEAE Sephadex	ug protein/ well	cpm/well X±SD	S.I. X±SD	cpm/well X±SD	S.I. X±SD	cpm/well X±SD	S.I. X±SD
Fraction 1 No NaCl	20.00	379±23	0.85±0.35	812±416	1.74±0.47	1074±509	1.98±0.86
	10.00	391±65	0.84±0.17	1423±1173	2.99±1.78	1945±2481	3.51±4.41
	5.00	491±115	1.10±0.46	1391±1120	3.04±1.8	683±224	1.26±0.36
	2.50	376±105	0.80±0.18	879±137	2.06±0.59	650±240	1.19±0.39
Fraction 2 0.05M Nacl	20.00	902±775	1.76±1.28	2686±2098	5.88±3.4	2157±267	4.01±0.48
	10.00	709±555	1.39±0.89	1971±399	5.05±3.13	1428±351	2.65±0.61
	5.00	1385±639	3.12±1.65	1690±203	4.30±2.51	1911±533	3.56±1.01
	2.50	1117±1004	2.19±1.67	2887±716	6.59±1.28	1661±1225	3.01±2.15

Fraction 3	20.00	263±21	0.58±0.19	1028±163	2.59±1.46	2237±1002	<u>4.13±1.75</u>
0.1M NaCl	10.00	231±65	0.48±0.07	928±314	2.06±0.25	1633±594	<u>3.01±1.0</u>
	5.00	207±44	0.44±0.05	787±365	1.74±0.47	1479±983	<u>2.74±1.76</u>
	2.50	200±41	0.42±0.04	618±252	1.40±0.41	1140±767	<u>2.09±1.36</u>
Fraction 4	20.00	251±51	0.58±0.30	1046±335	2.41±0.7	946±513	<u>2.75±0.92</u>
0.15M NaCl	10.00	260±87	0.54±0.09	1272±767	2.74±1.04	1118±349	<u>2.06±0.56</u>
	5.00	279±67	0.59±0.08	1442±821	3.27±1.42	915±362	<u>1.68±0.6</u>
	2.50	233±37	0.50±0.13	1335±783	2.83±0.96	930±414	<u>1.71±0.71</u>
Fraction 5	20.00	232±59	0.49±0.05	669±157	1.54±0.39	1306±365	2.42±0.62
0.2M NaCl	10.00	275±37	0.62±0.25	577±170	1.29±0.12	911±196	1.69±0.33
	5.00	252±64	0.54±0.11	660±228	1.45±0.1	753±240	1.38±0.38
	2.50	285±135	0.58±0.16	704±94	1.69±0.65	822±323	1.51±0.53
Fraction 6	20.00	233±84	0.48±0.10	873±566	1.81±0.76	909±123	1.68±0.17
0.25M NaCl	10.00	372±215	0.74±0.3	895±705	1.89±1.08	1043±406	1.97±0.88
	5.00	436±258	0.87±0.37	1053±427	2.54±1.24	971±201	1.82±0.48
	2.50	310±76	0.66±0.14	1308±489	3.24±1.82	773±206	1.43±0.32
Fraction 7	20.00	1004±881	2.03±1.42	1406±277	3.26±0.8	1413±638	2.60±1.08
0.3 M NaCl	10.00	2114±1366	4.14±1.92	2545±1170	5.52±1.16	1955±472	3.62±0.75
	5.00	2295±2915	4.19±1.03	2549±1291	5.71±2.02	931±179	1.74±0.41
	2.50	349±206	0.70±0.28	1479±1503	2.99±2.42	558±186	1.02±0.3
Concanavalin A	10.00	17443±9651	41.98±32.89	7180±2557	19.31±15.19	20051±12578	37.29±22.55
	5.00	30323±2242	67.32±21.79	14665±12253	31.21±19.01	33798±4946	62.89±8.16
Amastigote parasites	4 x 10 ⁶	1035±526	2.19±0.87	2327±974	5.17±1.23	5128±826	<u>9.52±1.21</u>
	2 x 10 ⁶	395±147	1±0.05	2427±1968	4.37±3.52	520±33	<u>0.90±0.5</u>
Culture medium		390±114	1.0±0	557±49	1.0±0.3	580±0	1.0±0

In Table 12, two groups of patients were evident before vaccination, specifically, one group with S.I. < 1.0 and another group with S.I. > 1.0. The group of patients cured after vaccination had markedly increased values when compared with either of these groups before vaccination. Results of the statistical analysis are as follows:

Group with S.I.< 1.0

	Before vaccination	After vaccination
Parameter		
Mean	1.150714	2.257857
# points	28	28
Std deviation	1.062052	.8876538
Std error	.200709	.1677508

Minimum .42 1.02
Maximum 4.19 4.13
Paired t test:
Mean difference = -1.107143 (Mean of paired differences)
5 95% confidence interval of the difference: -1.534381 to -.6799043
Two-tailed p value is < 0.0001 --- extremely significant-

Group with S.I.> 1.0

	Before vaccination	After vaccination
10 Parameter		
Mean	2.986429	2.257857
# points	28	28
Std deviation	1.504479	.8876538
Std error	.2843199	.1677508
15 Minimum	1.29	1.02
Maximum	6.59	4.13

Unpaired t test:
Mean difference = -.7285719 (Mean of B minus mean of A)
95% confidence interval of the difference: -1.3904 to -6.674413E-02
20 **Two-tailed p value is < 0.0316 --- significant-**

These results demonstrate that lymphocytes from both of the pre-vaccination groups are significantly stimulated by vaccination with any of the fractions of the *L.(V)brasiliensis* extract. Higher stimulation index was observed with fractions 3 and 4 as well as live
25 amastigotes.

Six fractions were separated from the particulate *L.(L)venezuelensis* extract (PMH16), the third component of the first-generation immunotherapeutic agent, after treatment of the respective amastigote parasites with TLCK and extraction with NP-40 as mentioned previously.

30 **TABLE 13: Peripheral blood mononuclear cells blastogenesis with fractions from *L.(L)venezuelensis* (PMH16) before and after vaccination.**

		BEFORE VACCINATION		BEFORE VACCINATION		CURED AFTER VACCINATION	
		n=5, S.I.< 1.0		n=2, S.I.> 1.0		n = 2	
DEAE Sephadex	ug protein/well	cpm/well X±SD	S.I. X±SD	cpm/well X±SD	S.I. X±SD	cpm/well X±SD	S.I. X±SD
Fraction 1 No NaCl	20.00	1617±1622	1.95±1.51	480±92	0.89±0.3	826±104	1.78±0.42
	10.00	1455±1241	1.82±1.03	737±57	1.36±0.72	518±74	1.11±0.62
	5.00	1222±905	1.57±0.66	488±75	0.90±0.43	551±42	1.1±0.63
	2.50	1376±1147	1.73±0.93	468±63	0.87±0.27	377±27	0.812±0.3

Fraction 2	20.00	1579±1259	1.77±1.39	1997±1965	1.86±1.05	2201±419	3.52±0.82
0.05M NaCl	10.00	1371±476	1.65±0.93	2163±489	2.65±1.02	1840±1895	2.41±1.89
	5.00	1003±455	1.11±0.48	1521±1235	1.52±0.46	1238±1093	1.68±0.97
	2.50	785±164	0.87±0.19	1398±1309	1.33±0.65	1259±1256	1.66±1.23
Fraction 3	20.00	896±358	0.98±0.36	1859±2160	1.61±1.41	3681±170	<u>6.08±2.25</u>
0.1M NaCl	10.00	948±594	1.02±0.53	4858±6397	3.92±4.67	4178±1306	<u>7.41±5.06</u>
	5.00	689±268	0.77±0.35	1299±1182	1.25±0.56	3802±1792	<u>6.96±5.61</u>
	2.50	707±302	0.77±0.29	1760±1967	1.55±1.23	2775±276	<u>4.53±1.45</u>
Fraction 4	20.00	848±401	0.89±0.25	1859±1316	1.93±0.3	2797±1204	<u>4.24±0.08</u>
0.15M NaCl	10.00	886±810	0.91±0.58	1930±95	2.49±1.35	3734±2376	<u>5.40±1.39</u>
	5.00	1105±1103	1.07±0.76	2024±402	2.81±2.08	1539±182	<u>2.63±1.37</u>
	2.50	826±479	0.90±0.49	1065±794	1.09±0.23	1151±442	<u>1.76±0.06</u>
Fraction 5	20.00	1087±618	0.91±0.53	2416±651	2.92±1.0	2612±1583	4.90±4.44
0.2M NaCl	10.00	848±601	1.14±1.26	1912±427	2.34±0.91	1648±165	2.80±1.41
	5.00	587±230	0.65±0.22	2092±108	2.78±1.75	2324±2119	4.60±5.13
	2.50	553±186	0.62±0.21	1434±842	1.56±0.1	1235±150	2.11±1.1
Fraction 6	20.00	767±15	1.14±0.42	129±15	2.40±0.57	1583±640	3.41±1.5
0.25M NaCl	10.00	515±91	0.74±0.16	852±22	1.58±0.63	1659±315	3.57±0.95
	5.00	374±31	0.55±0.17	577±46	1.07±0.38	592±92	1.27±0.47
	2.50	422±17	0.62±0.21	446±24	0.82±0.59	491±27	1.05±0.35
Concanavalin A	20.00	29329±13560	134±237	22781±8014	23.01±6.19	10028±4113	21.61±11.25
	10.00	34463±10198	40±17	48480±8611	66.96±48	24309±12540	52.39±36
	5.00	33799±7901	52±31	49409±7469	63.8±39	43290±6532	93.29±22.5
	2.50	35113±1040	52.28±18	42183±10112	58.2±19	35165±4526	75.78±36.5
Amastigote parasites	4 x 10 ⁶	1315±404	1.55±0.78	2933±429	3.22±0.11	2500±715	<u>5.38±1.2</u>
	2x 10 ⁶	1665±452	2.36±0.27			3032±1256	<u>6.5±3.4</u>
Culture medium		914±237	1.0±0.3	539±74	1.0±0.2	464±59	1.0±0

In Table 13 two groups of patients are evident before vaccination, specifically, one group with S.I.<1.0 and another group with S.I.>1.0. The group of patients cured after vaccination had markedly increased values when compared with either of these pre-vaccination groups.

5 Results of the statistical analysis are as follows:

Group with S.I. < 1.0

	Before vaccination	After vaccination
Parameter		
Mean	1.089583	3.205
5 # points	24	24
Std deviation	.4250269	1.938181
Std error	8.675825 E-02.	.3956296
Minimum	.55	.81
Maximum	1.95	7.41
10 Paired t test:		
	Mean difference = -2.115417 (Mean of paired differences)	
	95% confidence interval of the difference: -3.008944 to -1.22189	
	Two-tailed p value is < 0.0001 --- extremely significant-	

15 Group with S.I. > 1.0

	Before vaccination	After vaccination
Parameter		
Mean	1.814167	3.205
# points	24	24
20 Std deviation	.8092286	1.938181
Std error	.165183	.3956296
Minimum	.83	.81
Maximum	3.92	7.41
Unpaired t test:		
25 Mean difference = -.7285719 (Mean of B minus mean of A)		
	95% confidence interval of the difference: -1.3904 to -6.674413E-02	
	Two-tailed p value is < 0.0316 --- significant-	

These results demonstrate that lymphocytes from both pre-vaccination groups of patients are significantly stimulated by vaccination with any of the fractions of the *L.(L)venezuelensis* extract. Higher stimulation index was observed with fractions 3 and 4 as well as live amastigotes.

Seven fractions were separated from the *L.(L)amazonensis* extract (PMH8), the fourth component of the first-generation immunotherapeutic agent, after treatment of the respective amastigote parasites with TLCK and extraction with NP-40 as mentioned previously.

TABLE 14: Peripheral blood mononuclear cells blastogenesis with fractions from *L.(L)amazonensis* (PMH8), before and after vaccination.

		BEFORE VACCINATION		BEFORE VACCINATION		CURED AFTER VACCINATION	
		n = 4, S.I. < 1.0		n = 4, S.I. > 1.0		n = 4	
DEAE Sephadex	ug protein/well	cpm/well X±SD	S.I. X±SD	cpm/well X±SD	S.I. X±SD	cpm/well X±SD	S.I. X±SD

Fraction 1	20.00	450±22	0.84±0.1	265±22	1±0	1525±1374	1.48±0.97
No NaCl	10.00	371±19	0.70±0.35	285±45	1.07±0.3	1392±1222	1.95±1.27
	5.00	392±45	0.74±0.14	448±17	1.69±0.45	1211±584	1.79±0.46
	2.50	480±62	0.9±0.32	311±42	1.17±0.25	1152±733	1.67±0.71
Fraction 2	20.00	735±405	0.64±0.16	3576±4474	3.37±2.57	1614±1540	2.22±1.66
0.05M NaCl	10.00	574±356	0.59±0.26	1107±1066	1.38±0.07	1939±1297	2.24±1.35
	5.00	580±238	0.60±0.13	1181±1311	1.29±0.47	1569±970	2.28±1.10
	2.50	522±68	0.61±0.25	1173±1217	1.37±0.27	1180±1215	1.61±1.3
Fraction 3	20.00	885±928	0.84±0.61	1488±1524	1.76±0.3	1716±1355	<u>2.49±1.49</u>
0.1M NaCl	10.00	585±164	0.59±0.16	1582±285	3.29±2.71	2453±2095	<u>3.56±2.31</u>
	5.00	676±284	0.75±0.08	1073±850	1.53±0.35	807±423	<u>1.21±0.42</u>
	2.50	593±398	0.81±0.51	1267±1003	1.81±0.41	807±452	<u>1.20±0.45</u>
Fraction 4	20.00	733±64	1.38±0.6	349±15	1.31±0.4	1759±374	<u>2.80±0.74</u>
0.15M NaCl	10.00	428±26	0.84±0.2	1293±254	4.87±0.52	1424±152	<u>1.57±0.72</u>
	5.00	297±37	0.56±0.15	627±90	2.36±0.45	927±97	<u>1.49±0.4</u>
	2.50	374±29	0.70±0.14	397±26	1.49±0.65	939±559	<u>1.41±0.78</u>
Fraction 5	20.00	236±16	0.44±0.2	287±46	1.08±0.4	442±226	0.74±0.5
0.2M NaCl	10.00	383±45	0.72±0.15	231±26	0.87±0.22	421±127	0.67±0.24
	5.00	250±39	0.47±0.18	236±39	0.89±0.16	280±55	0.44±0.09
	2.50	276±52	0.52±0.27	302±11	1.13±0.45	334±43	0.54±0.17
Fraction 6	20.00	251±45	0.47±0.14	265±93	1±0	779±354	1.05±0.11
0.25M NaCl	10.00	284±17	0.53±0.21	250±42	0.94±0.4	679±235	1.03±0.24
	5.00	262±26	0.49±0.11	323±196	1.22±0.38	532±222	1.01±0.26
	2.50	264±32	0.49±0.12	298±29	1.12±0.6	450±236	0.73±0.48
Fraction 7	20.00	1038±453	2.03±0.5	522±125	1.97±0.5	1074±658	1.62±0.92
0.3 M NaCl	10.00	507±144	0.96±0.32	697±74	2.63±0.58	668±275	1.01±0.27
	5.00	395±61	0.74±0.37	611±85	2.30±0.45	898±674	1.37±0.9
	2.50	485±56	0.91±0.26	626±92	2.36±0.62	732±403	1.09±0.52
Concanavalin A	10	33179±9137	37.67±16.2	25676±13921	43.56±22.88	18975±10149	28.27±11.54
	5.00	31012±12118	36.31±7.42	39742±3747	86.32±75.86	17425±7521	26.31±8.18
Amastigote Parasites	4 x 10 ⁶	1775±702	2.15±0.67	2271±2564	2.44±1.0	3027±2268	<u>4.33±2.69</u>
Culture medium		510±89	1.00±0.1	265±59	1.0±0	529±67	1.0±0

In Table 14, two groups of patients are evident before vaccination, specifically, one group with S.I.<1.0 and another group with S.I.>1.0. The group of patients cured after vaccination had markedly increased values when compared with either of these pre-vaccination groups.

Results of the statistical analysis are as follows:

Group with S.I.< 1.0

	Before vaccination	After vaccination
Parameter		
5 Mean	.7007408	1.271786
# points	27	28
Std deviation	.2043736	.5430509
Std error	.0393317.	.102627
Minimum	.45	.47
10 Maximum	1.39	3.15
Unpaired t test:		
Mean difference = -.5710449 (Mean of paired differences)		
95% confidence interval of the difference: .3475174 to .7945725		
Two-tailed p value is < 0.0001 --- extremely significant-		

Group with S.I.> 1.0

	Before vaccination	After vaccination
Parameter		
20 Mean	1.726786	1.271786
# points	28	28
Std deviation	.9234719	.5430509
Std error	.1745198	.102627
Minimum	.88	.47
Maximum	4.88	3.15
25 Unpaired t test:		
Mean difference = -.4549999 (Mean of B minus mean of A)		
95% confidence interval of the difference: -.8608927 to -4.910712E-02		
Two-tailed p value is < 0.0287 --- significant-		

30 These results demonstrate that lymphocytes from both pre-vaccination groups of patients are significantly stimulated by vaccination with any of the fractions of the *L.(L)amazonensis* extract. Higher stimulation index was observed with fractions 3 and 4 as well as live amastigotes.

In summary, each of the blastogenesis experiments demonstrate that vaccination with
 35 any of the protein fractions from each of the leishmania species included in the first-generation immunotherapeutic agent, and particularly fractions 3 and 4, results in significant stimulation of lymphocytes. The stimulated lymphocytes produce cytokines that can inhibit the inflammatory response in psoriatic patients, thus inducing clinical remission of the psoriatic lesions.

Example 14

Humoral Immunity in Psoriatic Patients

TABLE 15: ELISA in psoriatic patients before and after vaccination. (O'Daly et al.1994 Acta Tropica 56:265-287)

Number of Patients	Immunotherapeutic agent Doses	Optical Density 405 nm (Average±S.D.)			
		La	Lv	Lb	Lch
36	0	0.21±0.20	0.40±0.18	0.37±0.22	0.35±0.18
13	1	0.12±0.00	0.21±0.09	0.22±0.10	0.19±0.07
18	2	0.37±0.27	0.35±0.16	0.32±0.17	0.33±0.14
17	3	0.47±0.22	0.38±0.15	0.41±0.20	0.36±0.10
12	4	0.41±0.28	0.30±0.11	0.22±0.09	0.26±0.03
12	6	0.38±0.27	0.34±0.18	0.36±0.05	0.30±0.01
	Active				
16	leishmaniasis	0.91±0.27	0.82±0.21	0.77±0.24	0.92±0.26

5 La: Leishmania amazonensis ; Lv:L.venezuelensis
Lb: L. brasiliensis ; Lch: L. chagasi

Sera from psoriasis patients were assayed before and after vaccination with an Enzyme Linked Immunosorbent Assay (ELISA), the results of which are shown in Table 15.

10 No difference in optical density values was observed between pre-vaccination and post-vaccination samples up to clinical remission of lesions after six doses of the first-generation immunotherapeutic agent. The cut-off point for a positive reaction was 0.5 units. The only positive sera belonged to samples from patients with active leishmaniasis. This demonstrates that the first-generation immunotherapeutic agent is not inducing Humoral Immunity or TH2

15 responses.

Example 15

Cellular Immunity in Psoriatic Patients

Table 16: Intradermic reaction to antigenic fractions in patients after clinical remission of psoriasis.

Parasite	Patients	IDR DIAMETER (mm)							P ¹
		CHROMATOGRAPHY FRACTIONS							
		1	2	3	4	5	6	7	
L.(L)chagasi	15	5.3±3.5	8.6±5.8	<u>21.7±5.0</u>	12.3±5.8	11.4±6.2	5.8±4.8	4.5±3.3	<0.0001
L.(V)brasiliensis	20	3.4±3.1	8.2±6.2	<u>14.9±5.5</u>	10.8±4.9	5.8±4.2	3.2±1.9	3.0±1.9	<0.0001

¹ Fraction 3 vs other fractions

The results of intradermic reaction assays for cellular immunity are shown in Table 16. The data indicate that the first-generation immunotherapeutic agent is inducing a TH1 response in cured psoriasis patients. Fraction 3 of the *L.(L)chagasi* and *L.(V)brasiliensis* antigenic components of the first-generation immunotherapeutic agent demonstrates the highest immunogenic activity *in vivo* with the intradermic reaction assay after clinical remission of lesions. Fraction 4 from either of these species also shows a high degree of activity.

Example 16

Single Blind Trial with Second-generation Immunotherapeutic agent

Containing Isolated Protein Antigenic Fractions

Table 17: Response to vaccination with second-generation immunotherapeutic agent.

Numbers of patients	Fraction	Numbers of Doses	Initial PASI	Final PASI	% Decrease in Final PASI
3	1	2.0±1.0	25.0±13.1	10.8±4.6	56.8
7	2	2.0±1.3	24.9±22.4	13.1±23.9	47.4
14	3	2.1±1.1	16.1±14.7	1.9±2.9	<u>88.2</u>
11	4	2.3±0.5	19.3±15.1	2.4±3.8	<u>87.6</u>
8	5	2.2±0.8	28.8±21.3	13.5±15.5	52.8
3	6	2.3±0.6	16.7±1.0	8.2±6.8	50.9

The effect of vaccination with the fractions of the second-generation immunotherapeutic agent on PASI values is shown in Table 17. Fractions 3 and 4 show the highest activity for clinical remission of psoriasis. Two doses of immunotherapeutic agent incorporating either of these fractions decrease the PASI by 88% of their initial values in patients before vaccination. These fractions also displayed the highest stimulation indexes in the *in vitro* blastogenesis experiments and the highest *in vivo* intradermic reaction (IDR)

diameter after vaccination in the patients cured of psoriasis.

Example 17

Identification and Characterization of Protein Fractions that Induce Clinical Remission of

5

Psoriatic Lesions

Peptides from acrylamide gels were transferred to nitrocellulose papers and analyzed at the ICBR Protein Chemistry CORE Facility at the University of Florida, Gainesville, Florida. HPLC was performed using a Hewlett Packard 1090 HPLC, digestion was performed with Endo-Lys-C, and amino acid analysis was performed using an ABI 494 Protein Sequencer. Amino acid sequence homology was searched using the BLAST program.

Table 18. Amino acid sequence of peptides.

Protein fraction		Band	Peptide number	Sequence	Peptide length	Homology with human proteins
3	82	2	12	YEDEINK	7	KERATIN TYPE II
			16	AQYEDIAQK	9	KERATIN TYPE II
	80	3	13	EIETYHNLLEGGQEDF	16	KERATIN TYPE I CITOSKELETAL
				AQYEDAIQK	9	KERATIN TYPE II
			10	YEDEINK	7	KERATIN TYPE II
	73	4	10	YEDEINK	7	KERATIN TYPE II
			12	AEAESLY	7	-
			13	NYSPYYNTIDDL	12	KERATIN TYPE I CITOSKELETAL
4	82	2	4	AEAESLYQSK	10	KERATIN TYPE II
			9	ATNAENEFV	9	KERATIN TYPE II
			22	XXYSELNRVIQRLRSI	16	KERATIN TYPE II
	80	3	18	EIETYHNLLEGGQEDF	16	KERATIN TYPE I CITOSKELETAL
			9	YEDEINK	7	KERATIN TYPE II
			11	AQYEDYAQ	8	KERATIN TYPE II
	73	4	8	YEDEINNK	8	-
			10	KYEDEINK	8	KERATIN TYPE II
			14	EIEQYLNLLASYLDF	16	KERATIN TYPE I CITOSKELETAL

			19	STMQELNSRLASYLDK	16	KERATIN TYPE I CITOSKELETAL
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Fraction 3 contained three bands after total reduction and alkylation as is known in the art. All but two of the peptide sequences showed homology to Keratin Type I or II human proteins. Fraction 4 showed similar results to fraction 3. This amastigote parasite keratin explains the effect of the immunotherapeutic agents of the present invention on psoriasis patients. Many authors have postulated that psoriasis is a disorder in human keratin from epidermal keratinocytes.

Example 18

Analysis of Peripheral Blood Lymphocytes with the Flow Cytometer

TABLE 19. Comparison of lymphocyte populations vs. healthy controls in psoriasis patients before treatment.

	0 DOSES n=95	CONTROLS n=49	p
CD4	30.7±12.8	40.8±9.6	<0.0001
CD8	20.3±9.3	28.4±9.7	<0.0001
CD8-CD4+	29±9.9	38.9±9.9	<0.0001
CD3	66.7±9.8	73.2±9.8	<0.0004
CD8+CD3+	13.1±7.3	19.5±8.6	<0.0001
HLA+	34.4±9.5	29.8±11.5	<0.0150
CD8+HLA-	11.9±5.9	14.7±7	<0.0129
IgE	6.7±3.8	4.8±2.2	<0.0061
IgG	0.8±0.5	1.2±0.6	<0.0026

All psoriasis patients, before treatment with the first-generation immunotherapeutic agent, showed peripheral blood lymphocyte populations significantly lower than normal healthy controls, with the exception of HLA and IgE markers, which were present at elevated levels.

TABLE 20: Comparison of lymphocyte populations vs. healthy controls in psoriasis patients with different degrees of disease severity following PASI values.

	PASI 1-9 p vs CONTROL		PASI 10-20 p vs CONTROL		PASI 21-65 p vs CONTROL	
	n=38	n=49	n=32	n=49	n=25	n=49
CD45	98.9±1.4	0.1283	99.0±0.1	0.1	98.9±1.2	0.1
CD4	36.6±9.2	0.0353	34.7±12.6	0.0334	22.4±10.2	<0.0001
CD8	23.1±8.6	0.0047	20.0±9.3	0.0008	18.0±6.7	<0.0001
CD8+CD4+	2.2±1.5	0.6253	1.7±1.3	0.8163	1.6±1.1	0.8379
CD8-CD4+	36.3±9.7	0.1838	28.6±10.4	0.0014	28.1±8.3	<0.0001
CD3	70.8±9.4	0.1100	66.3±10.9	0.0055	62.0±9.8	<0.0001
CD3+CD8-	57.1±10	0.0765	51.2±11.6	0.9311	51.3±7.9	0.9802

CD8+CD3+	15.5±8.5	0.0184	14.0±8.5	0.0100	12.8±6.9	0.0030
CD8+CD3-	6.8±3.6	0.4337	4.7±2.6	0.1182	4.4±3.9	0.0344
TCR	2.1±1	0.4337	2.1±1.7	0.3633	2.1±0.8	0.1441
HLA+	32.5±7.9	0.3389	32.8±7.7	0.2202	35.8±9.2	0.0424
CD8+HLA+	8.4±4.9	0.0574	7.6±5.2	0.0418	12.8±9.6	0.4227
CD8+HLA-	12.1±4.6	0.0483	12.6±5.8	0.1801	9.8±3.7	0.0039
CD19	7.4±3.6	0.8455	8.4±4.3	0.2806	8.0±3.5	0.5216

Peripheral blood lymphocyte populations were studied in psoriasis patients before treatment with the first-generation immunotherapeutic agent. Patients were distributed according to severity of the disease, tabulated according to PASI values. The results are shown in Table 20. As PASI values increased in psoriasis patients, peripheral blood lymphocyte populations of CD4+, CD8+, CD8-CD4+, CD3, CD8+CD3+, CD8+CD3-, CD8+HLA- decreased while populations of HLA+ increased relative to healthy controls. In the group with PASI 1-9, only four lymphocyte populations were lower than control values, while in the group with PASI 21-65, seven lymphocyte populations were lower than values for healthy controls. This suggests that lymphocytes migrate from peripheral blood to dermis and epidermis in the skin of psoriatic patients to induce the chronic inflammation characteristic of the disease.

TABLE 21: Comparison of lymphocyte populations in psoriasis patients with different degrees of disease severity.

	PASI	PASI	p	I.C. 95%	PASI	p	I.C. 95%
	[1-9]	[10-20]			[>20]		
CD4+	36.6±9.2	30.5±13.9	<0.4982		22.4±10.2	<0.0001	[-19.1 a -9.7]
CD8+	23.1±8.6	23.8±13.5	<0.1984		18.0±6.7	<0.039	[-9.3 a -1.8]
CD8+CD4+	2.2±1.5	2.0±2.1	<0.2139		1.6±1.1	<0.0001	[34.2 a 44.5]
CD8-CD4+	36.3±9.7	26.7±12.1	<0.0330	[-14.7 a -0.6]	23.1±8.3	<0.0001	[-20 a -7.5]
CD3	70.8±9.4	67.5±11.5	<0.0792		62.0±9.8	<0.0002	[-15.5 a -4.9]
CD3+CD8-	57.1±10.0	50±14.2	<0.0476	[-11.9 a 0.05]	51.3±7.9	<0.0118	[-13.9 a -1.8]
CD8+HLA-	12.1±4.6	13.0±6.1	<0.07337		9.8±3.7	<0.0310	[-4.4 a -0.21]
IGA+	5.1±2.9	7.4±3.6	<0.0443	[0.06 a 4.6]	10.5±7.0	<0.0001	[5.3 a 12.8]
IGD+	11.5±3.5	16.4±9	<0.0387	[0.17 a 6.25]	14.9±6.0	<0.1462	

There are significant differences in lymphocyte populations between patients with different PASI values. Comparison of 1-9 and 10-20 groups shows four lymphocyte populations with lower values in the group with a more severe psoriasis. Comparison between groups with

PASI 1-9 and PASI greater than 20 units showed seven lymphocyte populations with lower values in the group with severe psoriatic lesions. IgA+ lymphocytes were higher in the group with more severe disease.

5 **TABLE 22: Comparison of lymphocyte populations vs. healthy controls in psoriasis patients with total remission of lesions after more than 10 doses of first-generation immunotherapeutic agent.**

Cured patients > 10 DOSES of immunotherapeutic agent		p vs. CONTROL
	n=49	n=49
CD45	99.2±0.4	0.1283
CD45 RO	43.9±7.0	0.5406
CD4	43.2±9.4	0.7561
CD8	27.3±6.6	0.3985
CD8+CD4+	1.4±0.7	0.2537
CD8-CD4+	40.5±6.6	0.9923
CD3	70.0±9.5	0.063
CD3+CD8-	51.7±9.2	0.5583
CD8+CD3+	16.2±5.0	0.0634
HLA+	39.1±9.6	0.0108
CD8HLA+	14.9±7.1	0.0766
CD8HLA-	12.4±4.0	0.1113
CD19	10.9±4.9	0.0031

After clinical remission of lesions all peripheral blood lymphocyte populations returned to normal values, similar to healthy controls. Only HLA+ and CD19 lymphocyte
 10 populations had higher values than normal controls, probably because of lymphocyte stimulation after immunotherapeutic agent treatment.

The foregoing description of specific embodiments is merely illustrative, and various modifications may be made without deviating from the spirit and scope of the present invention, which is limited only by the following claims.